TARGETED PEPTIDES FROM MAJOR ROYAL JELLY PROTEINS AS CHEMICAL MARKERS TO DISTINGUISH *Mimosa scabrella* Bentham HONEYDEW HONEY

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RESUMO – Este estudo analisou as principais proteínas da geleia real (PGR) em méis de melato de bracatinga (*Mimosa scabrella* Bentham) e méis florais do Brasil e da Alemanha, utilizando espectrometria de massas de alta resolução para identificar peptídeos marcadores. A abordagem envolveu a digestão tríptica das PGR do mel, seguida da quantificação direcionada de peptídeos por LC-QqQ-MS/MS. Entre os peptídeos marcadores selecionados, o GVPSSLNVISEK da PGR5 foi encontrado em níveis mais altos no mel de melato da bracatinga. Embora este peptídeo tenha potencial como marcador de autenticidade deste mel, ele não pode ser utilizado como único critério de classificação. Além disso, discrepâncias em relação a análises anteriores de PGR em méis de melato da bracatinga destacaram o impacto das condições de armazenamento nas alterações do conteúdo proteico.

ABSTRACT – This study analyzed major royal jelly proteins (MRJP) in bracatinga (*Mimosa scabrella* Bentham) honeydew honeys and floral honeys from Brazil and Germany using high-resolution mass spectrometry to identify marker peptides. The approach involved tryptic digestion of honey MRJP and targeted peptide quantification via LC-QqQ-MS/MS. Among the selected marker peptides, GVPSSLNVISEK from MRJP5 was found at higher levels in bracatinga honeydew honey. While this peptide holds potential as a marker for the authentication of this honey, it cannot be used as a sole classification criterion. Additionally, discrepancies with previous MRJP analyses in bracatinga honeydew honeys highlighted the impact of storage conditions on protein content changes.

PALAVRAS-CHAVE: LC-MS/MS; MRJP; proteômica; mel de melato da bracatinga.

KEYWORDS: LC-MS/MS; MRJP; proteomics; bracatinga honeydew honey.

1. INTRODUCTION

Bracatinga honeydew honey (BHH) is a dark honey-like produced in South Brazil. BHH originates from the exudates of scale insects (*Stigmacoccus paranaensis* Foldi), which feed on the sap of bracatinga trees (*Mimosa scabrella* Bentham). *Apis mellifera bees* collect exudates and produce honey every two years – according to the cochineal (scale insect) life cycle. This honey has a Protected Designation of Origin (PDO) under the name "Mel de Melato da Bracatinga do Planalto Sul Brasileiro" (INPI, 2021; Silva et al., 2022).

Expanding the knowledge of this product is essential for identifying chemical markers that support its authenticity assessment and enhance the value of this unique natural resource. However, accurately identifying bracatinga honeydew honey remains challenging due to its compositional variability and the absence of definitive authenticity markers. While the presence of major royal jelly proteins (MRJP) in BHH has been demonstrated using proteomic approaches (Silva et al., 2021), with a peptide from MRJP4 classified as a potential marker, the possible variations in peptide patterns could help to understand if they are reliable chemical markers.

Notwithstanding that finding a reliable marker for honey is difficult due to the complex composition, this work aimed to assess if peptides reflect the genetically relevant major proteins with the possible influence of the environmental or storage conditions and, therefore, help to provide a good basis to evaluate the authenticity of BHH.

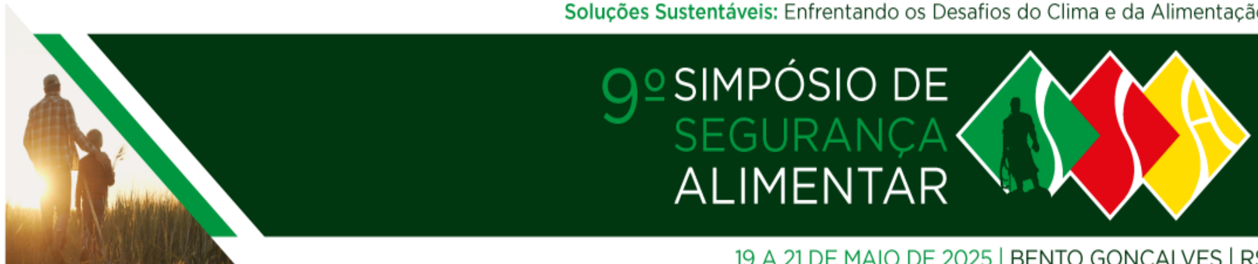
2. MATERIAL AND METHODS

2.1 Samples

Thirty (n = 30) honey samples were analyzed. Among these, 16 were bracatinga honeydew honeys — 8 labeled as BCH, acquired commercially and processed by the producer, and the remaining 8 were referred to as BHH, obtained by draining honeycombs in the laboratory. The remaining 14 honeys were classified as commercially available floral honeys, with 8 originating from Brazil (FH-B) and 6 acquired from a German supermarket (FH-G). All honey samples were collected in 2020 and stored in air-tight polypropylene tubes at room temperature (RT) until the analysis.

2.2 Extraction and digestion of honey proteins

The honey samples were prepared by adapting the procedure established by Silva et al. (2021). Each honey sample (500 ± 0.1 mg) was dissolved in 1 mL of extraction buffer (4 M urea, 5 mM dithiothreitol (DTT), 100 mM ammonium bicarbonate), and shaken at 80 rpm for 10 min. Afterward, 0.4 mL of the supernatant was transferred to 10 mL SP-D Plus pressure vessels (CEM Corporation,



Matthews, North Caroline, USA). Disulfide bridges in the proteins were reduced and alkylated by adding 10 µL of 0.5 mM iodoacetamide (IAA), followed by incubation in a water bath at 50 °C for 20 min in the dark.

The proteins were digested with trypsin by adding 10 µL of standard peptide GWGG (1 µg mL^{-1}), 164 µL of digestion buffer (18% acetonitrile (v:v) and 100 mM ammonium bicarbonate) and 20 µL of trypsin to each sample. The digestion was done in a microwave reactor (Discover SP D, CEM Corporation, Matthews, North Caroline, USA) at 50°C for 10 min. The reaction was terminated by adding 15 µL of 40% formic acid. The supernatant (400 µL) was transferred to a vial and stored at 4°C until measurement. Tryptic peptides were measured and further fragmented by HPLC-MS/MS to facilitate the identification of proteins (Huynh et al., 2009). Every analyzed protein included at least a quantifier and a qualifier peptide for identification purposes. A quantifier peptide is unique for a protein, while a qualifier peptide is found in the target protein but can occur in other proteins as well (Van Den Broek et al., 2020).

2.3 LC-QqQ-MS/MS analysis of major royal jelly proteins (MRJP)

The MRJP 1, 2, 3, 4, 5, and 7 were analyzed employing the Agilent Infinity 1260 system (Agilent Technologies, Santa Clara, CA, USA) with binary pump (G7112B), multicolumn thermostat (G7116A) and autosampler (G7129A) equipped with an Agilent G6470A Series Triple Quad LC-MS (Agilent Technologies, Waldbronn, Germany) coupled with an electrospray source. The separation of peptides was performed with a Kinetex C8 analytical column (2.6 µm, 100 Å, 150 × 4.60 mm; Phenomenex, Torrance, CA, USA) and a mobile phase comprising of eluent A (0.1% formic acid) and eluent B (acetonitrile) at a flow rate of 0.5 mL min^{-1}. Detection was performed in the multiple reaction monitoring (MRM) mode with gradient conditions adapted from Silva et al. (2021). The potentially signal-interfering substances were removed by directing the mobile phase into the waste for the first 5 min to remove the high quantities of sugars that are eluted by the 95% water content of the gradient. The post-run time was 4 min. The injection volume was 10 µL.

2.4 Data analysis

The assays were performed in three independent replicates, and the results were expressed as mean ± standard deviation. The results were analyzed using one-way ANOVA followed by Tukey's post hoc test using STATISTICA 13.5 (TIBCO Software Inc., Palo Alto, CA, USA), with a p-value less than 0.05 being considered significant ($p < 0.05$).

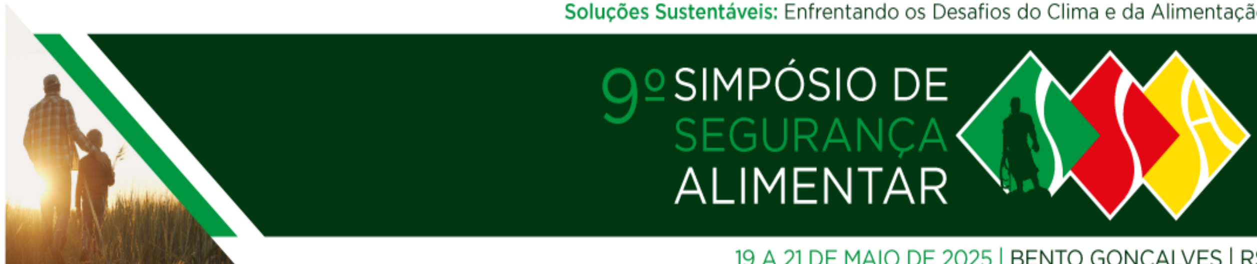
3. RESULTS AND DISCUSSION

A proteomics approach was used to identify potential MJRP peptide markers specific for BHH (Table 1). The analysis of the respective peptides of MRJP2, 3, 4, and 7 exhibited no significant difference between bracatinga honeydew and floral honeys. The MRJP4 quantifier peptide QNIDVVAR has been proposed as a marker to differentiate bracatinga honeydew honeys from floral honeys, because this peptide was found in significantly higher concentrations in BHH than in floral honeys (Silva et al., 2021). However, these results could not be confirmed. QNIDVVAR did appear in higher concentrations in BHH than FH-B but did not differ from FH-G. Furthermore, as stated by Silva et al. (2021), no difference was observed between BCH and floral honeys. Therefore, using the peptide QNIDVVAR is not viable for authentication, especially if BCH is involved.

Table 1. Average MRJP content (peak area/µ honey injected) in honeys by HPLC-MS/MS.

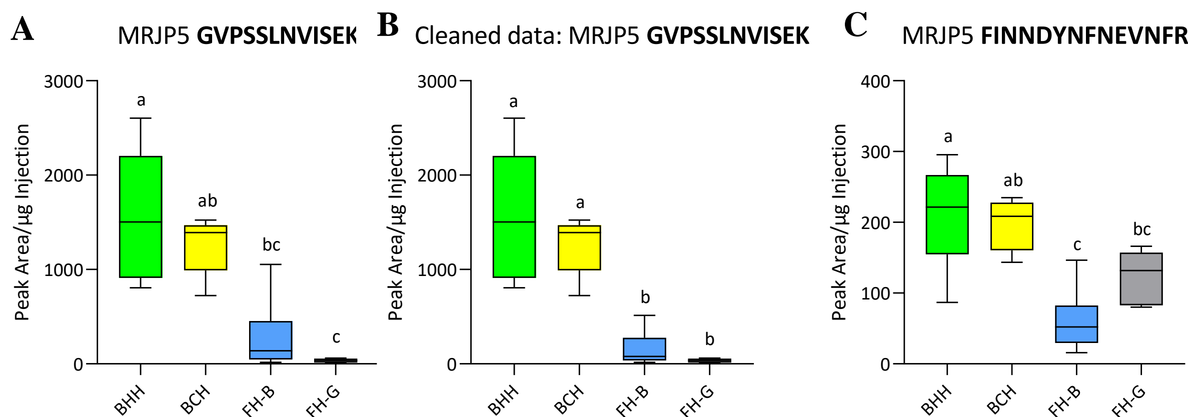
	BHH	**BCH**	**FH-B**	**FH-G**
MRJP1				
SLPILHEWK	2638.5 ± 698.3^{a}	2624.2 ± 827.8^{a}	1742.8 ± 1879.7^{a}	3322.9 ± 1268.8^{a}
LLTFDLTTSQLLK	2207.1 ± 1949.6^{a}	1345.5 ± 1415.6^{a}	165.7 ± 122.9^{b}	1107.1 ± 876.0^{a}
EALPHVPIFDR*	287.0 ± 113.3^{b}	370.8 ± 170.2^{b}	385.9 ± 210.7^{b}	3403.5 ± 1521.8^{a}
MRJP2				
TFVTILR	1254.6 ± 377.5^{a}	1180.0 ± 264.0^{ab}	891.3 ± 581.8^{ab}	384.0 ± 121.0^{b}
LHVFDLK*	572.4 ± 922.5^{ab}	579.0 ± 1172.1^{ab}	90.9 ± 100.7^{b}	807.3 ± 723.3^{a}
ILGANVK*	483.5 ± 150.8^{ab}	529.3 ± 95.7^{ab}	336.4 ± 229.1^{b}	867.7 ± 691.2^{a}
MRJP3				
VIYEWK	158.8 ± 52.4^{b}	140.0 ± 56.3^{b}	128.2 ± 115.5^{b}	1345.5 ± 884.7^{a}
NYPFDVDR	222.3 ± 135.2^{ab}	175.3 ± 61.0^{b}	99.1 ± 79.5^{b}	823.7 ± 392.7^{a}
MRJP4				
QAAIQSGEYDR	82.7 ± 26.4^{a}	71.9 ± 21.0^{ab}	42.5 ± 29.6^{b}	83.4 ± 33.0^{a}
QNIDVVAR*	908.0 ± 292.4^{ab}	771.0 ± 178.9^{ab}	517.2 ± 318.2^{b}	1060.7 ± 431.1^{a}
MRJP5				
GVPSSLNVISEK	1598.4 ± 686.5^{a}	1257.7 ± 290.1^{a}	280.9 ± 353.4^{b}	34.9 ± 21.3^{b}
FINNDYNFNEVNFR*	209.7 ± 68.8^{a}	198.1 ± 35.0^{ab}	61.0 ± 42.1^{c}	122.3 ± 38.2^{bc}
MRJP7				
LLQPYPDWSWTK*	205.5 ± 65.2^{b}	174.1 ± 47.1^{b}	163.3 ± 142.2^{b}	453.2 ± 151.0^{a}
LLVFDLNSSQLIK	247.0 ± 185.6^{a}	127.1 ± 140.3^{ab}	30.4 ± 20.5^{b}	133.4 ± 130.1^{ab}
ILNNDLNFNDINFR	358.1 ± 112.0^{a}	236.9 ± 23.6^{a}	63.7 ± 54.2^{b}	181.3 ± 88.7^{ab}

Results are expressed as mean ± standard deviation. a, b, and c mark significant differences ($p < 0.05$) between the honeys identified by one-way ANOVA. BHH, bracatinga honeydew honey; BCH, bracatinga honeydew honey commercial; FH-B, Brazilian floral honey; FH-G, German floral honey; MRJP, major royal jelly protein. *Quantifier peptide.



For MRJP5, the quantifier peptide FINNDYNFNEVNFR was higher in BHH than in FH-B and FH-G but showed no difference between BCH and FH-G. In contrast to the results of Silva et al. (2021), who could not detect the qualifier peptide GVPSSLNVISEK and consequently discarded it, in this work, GVPSSLNVISEK has shown a fair intensity and potential as a marker (Figure 1). Although this peptide is not specific to MRJP5, it is only found in bee-specific proteins (MRJP5, MRJP6, MRJP7, and two uncharacterized proteins), according to a peptide search in UniProt database (https://www.uniprot.org/). However, since a floral honey sample FH-B did exhibit a high concentration of GVPSSLNVISEK, it cannot serve as the sole marker for authentication. Nonetheless, an elevated amount of this peptide indicates bracatinga honeydew honeys, which, combined with other biomarkers, may be a valuable component for the authentication process.

Figure 1: Intensities of the peptides selected for MRJP 5 analysis. MRJP: Major royal jelly protein; BHH: Bracatinga honeydew honey; BCH: Bracatinga honeydew honey commercial; FH-B: Brazilian floral honey; FH-G: German floral honey; a, b and c mark significant differences ($p < 0.05$) between the honeys identified by a one-way ANOVA



The storage conditions of the honey samples may explain the divergent results compared to the previous work (Silva et al., 2021). Prolonged storage of honeys (6 months) at RT can lead to a decrease in protein content (BRUDZYNSKI et al., 2013). The reason for this may be the development of poorly soluble or insoluble quinone-protein complexes in the stored honeys, which can form through the oxidation of polyphenols to quinones. An increase in protein-phenol complexes occurs in honey when stored over long periods at RT, which in return reduces the peptide yield after tryptic digestion.

4. CONCLUSION

A proteomic strategy was applied to bracatinga honeydew honey and floral honeys from Brazil and Germany to identify MRJP peptides as potential chemical markers for bracatinga honeydew honey, contributing to its authenticity. The results showed that only the peptide GVPSSLNVISEK from MRJP5 could be considered a marker, but not isolated, requiring additional chemical markers for accurate authentication. Moreover, the findings differed from a previous study, suggesting that storage conditions influence protein concentrations in honey, likely due to complex formation with quinones. Further studies should evaluate the impact of storage on honey proteins and identify multiple target markers for more reliable differentiation.

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